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Review article

ANTITUMOR EFFECT OF SNAKE VENOMS

R. J. DA SILVA[1] ✉ , D. FECCHIO[2] , B. BARRAVIERA[1,3]

1 Center for the Study of Venoms and Venomous Animals - CEVAP-UNESP, State of São Paulo, Brazil; **2** Department of Pathology of the School of Medicine of Botucatu, State of São Paulo, Brazil, **3** Department of Tropical Diseases of the School of Medicine of Botucatu, State of São Paulo, Brazil.

ABSTRACT. The search for biological antitumor agents has been pursued for over half a century. Snake venom has been shown to possess a wide spectrum of biological activities. The objectives of the present review are to evaluate the existing controversies on this subject published in a number of papers and to propose probable explanations for the phenomena observed. We reported our results obtained in a study, in which we evaluated the action of the venoms of *Crotalus durissus terrificus* and *Bothrops jararaca* on Ehrlich ascites tumor cells. We noticed an important antitumor effect, mainly with *Bothrops jararaca* venom, as well as an increase in the functional activity of macrophages. We also observed an increase in the number of mononuclear and polymorphonuclear cells with *Bothrops jararaca* venom. Considering these findings, we postulate that both *Bothrops jararaca* and *Crotalus durissus terrificus* venoms can act directly on tumor cells. In addition, we propose an indirect mechanism, based on the stimulation of the inflammatory response, to inhibit tumor growth and to promote its rejection.

☑ **KEY WORDS:** cancer, inflammation, venom, snakes, *Crotalus durissus terrificus, Bothrops jararaca.*

INTRODUCTION

The search for antitumor agents has aroused the interest of scientists since the beginning of this century. In 1933, Calmette *et al.*(**8**). reported an antitumor effect of the venom of *Naja* sp. on adenocarcinoma cells. From that time on, a number of papers have been published on the subject and controversies still exist (**3,5,6,8,9,10,15**) (**20,25,27,29,30,34**).

Yoshikura *et al.*(**34**) investigated the action of crude venom of *Trimeresurus flavoviridis* and that of two hemorrhagic principles isolated from this venom HR1 and HR2. These authors carried out *in vitro* studies using cultures of HeLa cells, the mouse embryo secondary culture cells, MLg cells originated from the lung of newborn mice and fibroblast from human embryos. The authors(**34**) observed that both the crude venom and the hemorrhagic principle HR2 caused morphological changes in various cell lines tested, without promoting cellular death. After venom removal, the cells grew normally again. Hemorrhagic principle HR1 did not exert any effect on the cell lines used.

Later on, Bragança *et al.*(**6**) demonstrated that cytotoxin isolated from *Naja naja* venom

was cytotoxic to Yoshida sarcoma cells implanted in mice, but was non-toxic to the experimental animals.

Patel *et al.*(**27**) also investigated the antitumor properties of *Naja naja* venom. These authors (**27**) carried out research with a fraction (cytotoxin) isolated from this venom to evaluate its effects on Yoshida sarcoma cells. The experiments conducted *in vitro* showed that cytotoxin acted on the tumor cells first leading to swelling of the cell to almost double its original volume. It was followed by shrinkage which was accompanied by a release of cytoplasmic RNA and proteins and destruction of tumor cells. The authors (**27**) also reported that the destruction of tumor cells was influenced by the concentration of sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions, high temperature and pH.

Bragança(**5**) also reported cytotoxic effects *in vivo* of fraction P6 isolated from *Naja* sp. venom on Yoshida sarcoma, mouse fibrosarcoma and Ehrlich ascites tumor cells. The author(**5**) reported that fraction P6 did not affect erythrocytes, leukocytes and lymphocytes.

Cotte *et al.*(**10**) studied the action *in vivo* of the venoms of *Bothrops atrox, Bothrops venezuelae* and *Crotalus durissus terrificus* on lines of tumor cells from human breast carcinoma, an epidermoid carcinoma of the uterine cervix and from carcinoma of the larynx. They(**10**) also evaluated the effects of these venoms on lines of normal chick embryo, human kidney and monkey kidney cells and observed a marked cytolytic effect of the three venoms upon the cells.

Markland Jr.(**25**) investigated the effects *in vitro* and *in vivo* of the thrombin-like enzyme (crotalase) from the venom of *Crotalus adamanteus* on the growth of mouse B16 melanoma cells. The results obtained by this author(**25**) suggested that crotalase significantly retarded tumor growth *in vivo*, did not present cytotoxic or cytostatic effects on cells *in vivo* and had no significant effect on increasing the survival time of the animals. The author(**25**) also reported that 50% of the crotalase-treated animals did not develop the tumor, while in the control group 10% of the animals did not develop the tumor.

Chaim-Matyas and Ovadia(**9**) screened *in vitro* and *in vivo* snake venoms of the families Elapidae (*Naja naja atra, Naja naja naja* and *Naja nigricolis*), Crotalidae (*Trimeresurus flavoviridis*) and Viperidae (*Bitis arietans, Echis colorata, Pseudocerastes fieldi, Vipera ammodytes, Vipera bornmuelleri, Vipera palaestinae, Vipera radii, Vipera russelli* and *Vipera xanthina*) for cytotoxicity towards B16F10 melanoma and mouse chondrosarcoma cells.These authors(**9**) also observed cytotoxic activity of these venoms.

Hernandez-Plata *et al.*(**15**) reported the results obtained with the treatment of spontaneous sarcoma cells of female rats with fractions of *Crotalus durissus terrificus* venom, crotamine and crotoxin complex A and B. Their results regarding both tumor regression and increase in the animals' survival time were considered excellent.

Recently, Lipps(**20**) evaluated the antitumor effect of snake venoms of two snake species *Crotalus atrox* and *Naja naja kaouthia*. The author(**20**) fractionated both venoms, tested all fractions of each venom for their biological activity on mouse myeloma cells, and isolated from each venom the fraction with maximum cytolytic activity against tumor cells. These fractions were called atroporin and kaotree isolated from *Crotalus atrox* and *Naja naja kaouthia* venoms, respectively. Then, atroporin and kaotree were further purified and tested, alone or in combination, showing killing effects on various types of human (breast, colon, liver and ovary) and animal cancer cells (mouse myeloma, pheochromocytoma, rat adrenal and monkey kidney) and on normal mouse kidney, liver, spleen cells and erythrocytes. Lipps(**20**) conducted studies *in vitro* to evaluate atroporin and kaotree cytolytic effect on all tumor cell types used, and *in vivo*, to assess their properties of preventing tumor growth, as well as those of causing regression of growing tumors. The combination of atroporin and kaotree exhibited higher antitumor activity than that of each fraction alone. Furthermore, Lipps(**20**) noted that neither atroporin nor kaotree showed cytotoxic effect on normal cells used in the experiment.

In contrast to the findings that have been presented so far, Shiau-Lin *et al.*(**29**) studied the snake venoms of the species *Naja*, and failed to obtain satisfactory results in tumor growth control. These authors(**29**) used cytotoxin from *Naja naja* venom, and cardiotoxin from *Naja naja atra* venom, to compare *in vitro* their effect on Ehrlich ascites tumor cells, but did not observe any carcinolytic activity.

Iwaguchi *et al.*(**17**) tested *in vivo* and *in vitro* the effects of cytotoxin I and II from *Naja naja* venom on tumor cells such as Yoshida sarcoma, and also toward normal cells such as erythrocytes, spleen and peritoneal exudate from rats. These authors(**17**) did not observe any antitumor effect *in vivo*. However, *in vitro*, they noticed a higher cytolytic effect on tumor cells than on normal cells.

Baldi *et al.*(**3**) evaluated the effect of *Naja naja siamensis* venom and that of crotoxin complex A and B from *Crotalus durissus terrificus* venom on tumor cells such as human metastatic breast adenocarcinoma, and murine sarcoma 180, Ehrlich ascites tumor and

breast carcinoma. They carried out experiments both *in vitro* and *in vivo*, but failed to see any antitumor effect of both tested substances.

Taking into consideration what has been presented so far, we can conclude that there are controversies among the authors in regard to the action of snake venoms on tumor growth.

We have recently investigated(**30**) the effect of *Crotalus durissus terrificus* and *Bothrops jararaca* venoms on the growth of Ehrlich ascites tumor cells in mice. We noted a major antitumor effect, mainly with the *B. jararaca*-venom-treated groups. Also, we detected an increase in the functional activity of macrophages in the groups treated with both venoms, as well as an increase in the number of mononuclear and polymorphonuclear cells in the *B. jararaca*-venom-treated groups.

The exact mechanisms which cause tumor regression in experimental animals after treatment with crude snake venom and/or any fraction of this venom still are unknown.

According to Lipps(**20**), certain fractions isolated from snake venoms revealed a direct cytolytic activity on tumor cells. Markland(**25**) upon conducting research on crotalase proposed the following mechanism to explain regression of tumor cells. Malignant cells would produce a microenvironment around them, using substances of the host itself which protected them against defensive responses of the immune system. This microenvironment would be composed of fibrin deposits from the nearby blood vessels. Crotalase apparently destroyed the microenviroment produced by the tumor cells attacking fibrinogen directly, which led to the formation of soluble fibrin monomers or abnormal fibrin microclots that were rapidly removed by secondary activation of the fibrinolytic system. Thus, immunological masking would not have occurred, which would leave tumor cells unprotected.

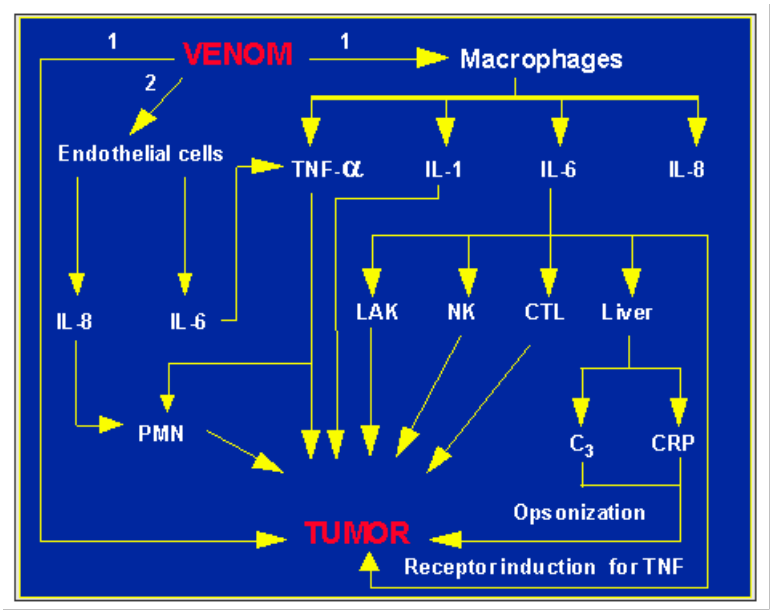
Both the mechanism proposed by Lipps(**20**) and that proposed by Markland(**25**) may be important. However, we believe that these are not the only mechanisms which act upon experimental tumors.

It is worth considering that tumor cells are capable of inhibiting the hosts inflammatory response, which was first demonstrated by Mahoney and Leighton(**24**). These authors(**24**) reported that tumor tissues of an individual presented a much less intense inflammatory response than that of a normal tissue of the same individual. Fauve *et al.*(**11**) explained that the implantation of teratocarcinoma in mice diminished the intensity of the inflammatory response at the implantation site. They also showed that the diminished intensity of the inflammatory response was due to the release of low-molecular-weight substances which inhibited the influx of polymorphonuclear leukocytes. Snyderman *et al.*(**32,33**) stated that tumor cells affected the influx of macrophages *in vivo* and chemotaxis *in vitro*, without altering the migration capacity of polymorphonuclear leukocytes. In addition, Fechio *et al.*(**12**) reported that Ehrlich ascites tumor cells induced a poor inflammatory response in mice.

Interaction of snake venoms and animal cells, as well as the result of such interaction still remain to be further explained. Thus, this interaction is likely to cause the release of certain chemical mediators of the inflammatory response which might compensate for the absence of this tumor-induced response(**11,12,24,32,33**). Snake venoms might, thereby, act on tumor cells by either direct or indirect mechanisms.

Figure 1 illustrates the hypothetical effect of *Crotalus durissus terrificus* and *Bothrops jararaca* venom on Ehrlich ascites tumor cells. The venoms may have a lytic action on tumor cells, as was demonstrated by Lipps(**20**), and as was shown in Figure 1. Conversely, the possible indirect mechanisms will be discussed below.

FIGURE 1. Hypothetical mechanisms involved in the rejection of tumor cells.
1. *Crotalus durissus terrificus* and *Bothrops jararaca*; **2.** *Bothrops jararaca*; IL = interleukin; TNF-α = tumor necrosis factor-alpha; PMN = polymorphonuclear leukocytes; LAK = lymphokine-activated killer cells; NK = natural killer cells; CTL = cytotoxic T lymphocytes; CRP = C-reactive protein; C3 = complement factor C3.



According to Abbas *et al.*(**1**), macrophages play an essential role in the body immune system. When stimulated by certain antigens, macrophages produce and release several mediators of the inflammatory response such as the tumor necrosis factor-alpha (TNF-α) and the interleukins (IL), mainly IL-1, IL-6 and IL-8.

We(**30**) noted that macrophages of animals inoculated with *C. durissus terrificus* and *B. jararaca* venoms had an increased percentage of spreading. Macrophages presented increasing in size with many cytoplasmic vacuoles, which suggested that they were active and likely to be producing and releasing their metabolic products. Activated macrophages may release substances such as TNF-α , IL-1, IL-6 and IL-8 to the extracellular medium.

It has been described in literature(**2**) that, among other functions, TNF-α plays an important role in tumor rejection. The mechanism to explain TNF-α cytotoxicity is rather complex and involves the triggering of complement G-protein and the increase in the cytoplasmic content of cyclic AMP. Production of free radicals of oxygen and arachidonic acid metabolites has also been described. All these events together might lead to tumor cell death.

Kimura *et al.*(**18**) evaluated the effects of the treatment of human thyroid carcinoma using IL-1. The authors observed an inhibition of tumor cell growth after administration of IL-1, and they suggested that such inhibition was due partly to suppression of tumor cell messenger RNA. In addition, the authors(**18**) postulated that IL-1 exerted an antiproliferative effect on different lines of malignant cells such as those of breast carcinoma, cervical carcinoma, the myelogenous leukemia cells and melanoma.

Lomonte *et al.*(**21,22**) investigated the inflammatory response of mice that were subcutaneously injected with *Bothrops asper* venom. The authors(**21,22**) detected a rise in serum IL-6 concentration between 3 and 6h after venom inoculation. In contrast, they(**21,22**) did not detect either serum TNF-α or IL-1. These authors(**21,22**) also noted edema and polymorphonuclear leukocyte infiltrate between 6 and 24h after venom inoculation. Following this period, there was a significant predominance of macrophages.

A similar finding was described by Barraviera *et al.*(**4**) who studied patients bitten by *Crotalus* and *Bothrops* snakes, upon evaluating the seric level of IL-1, IL-6, IL-8 and TNF-α in the first 5 days after envenomation. The authors(**4**) reported that IL-6 was enhanced in all the patients bitten by *Crotalus* snakes and in 75% of the patients bitten by *Bothrops* snakes. IL-8 was higher in 62.5% and in 12% of the patients bitten by *Crotalus* and *Bothrops* snakes, respectively. IL-1 was detectable in only 37.5% of the victims bitten by *Crotalus* snakes, while TNF-α was not detected.

The researchs of Lomonte *et al.*(**21,22**) and that of Barraviera *et al.*(**4**) revealed that snake venoms induce the release of chemical mediators of the inflammatory response, especially IL-6.

Several authors(**7,14,16,19**)_(**23,26,28,31**) have shown that IL-6 plays a major role in tumor rejection, and that the precise mechanism by which IL-6 rejects tumor is quite complex.

IL-6 has also been described as a modulator of the function of lymphokine-activated killer cells (LAK) which are human peripheral blood mononuclear cells. Gallagher *et al.*(**14**)

reported that when IL-6 was included with IL-2 during the induction phase of the LAK cells, it enhanced the lytic activity of these cells on tumor cells. However, the authors(**14**) postulated that the rate of tumor cell rejection was not due to an increase in the number of LAK cells, but rather to an enhancement of LAK capacity to lyse tumor cells.

Iho *et al.*(**16**) reported that LAK cells could lyse tumor cells including natural killer (NK)-resistant cell lines and autologous tumor cells. In accordance with Gallagher *et al.*(**14**), these authors(**16**) stated that IL-6 enhanced the function of IL-2-activated LAK cells released by T lymphocytes.

IL-6 has also been described as a modulator of NK cell function which is characterized by the capacity of the non-immunized lymphoid cells to recognize and lyse a wide variety of tumor cells. According to Luger *et al.*(**23**), IL-6 augmented the cytotoxic activity of NK cells (CD^{3-}) promoting an efficient cellular lysis. The authors(**23**) added that the activation of the NK (CD^{3-}) cell cytotoxicity was IL-2-modulated since IL-6 showed itself to be a potent inducer of IL-2 production. Similar finding was reported by Smith *et al.*(**31**). These authors(**31**) demonstrated that high concentrations of IL-2 could induce peripheral blood T cell pore-forming protein (PFP) which lysed tumor cells. However, they(**31**) also reported that the levels of IL-2 needed to induce these effects on T cells could be significantly reduced in the presence of IL-6.

Another major function of IL-6 is that of cytotoxic cell inducer. Kitahara *et al.*(**19**) and Okada *et al.*(**26**) revealed that *in vitro* administration of IL-6 increased the lysis rate of tumor cells, and its *in vivo* administration augmented the cure rate of animals bearing tumor cells of different lines by induction of tumor-specific cytotoxic T lymphocytes (CTL). In addition, the authors(**19,26**) demonstrated that administration of IL-6 did not exert any direct cytostatic and/or cytolytic effect on tumor cells, and that the presence of IL-2 was essential in early phases of CTL induction, while that of IL-6 was essential in late phases.

Cairns *et al.*(**7**) described IL-6 as a regulator of TNF-α activity. These authors(**7**) showed that low concentrations of IL-6 enhanced the cytotoxic effects of TNF-α in human lymphoma cells in a dose-dependent manner. The authors(**7**) suggested that this enhanced activity might have occurred due to IL-6 capacity to express the membrane receptors for TNF-α .

Scheid *et al.*(**28**) further reported IL-6 activity in the liver by inducing the release of acute-phase proteins such as C-reactive protein both *in vitro* and *in vivo*. According to these authors(**28**), IL-6 also promoted a rise in seric levels of complement factor C3. Both the C-reactive protein and the complement factor C3 served as opsonins, binding to tumor cell receptors and increasing cytotoxicity exerted by NK cells and macrophages.

Venoms can also act on the endothelial cells promoting the release of IL-6 and IL-8 (**4,21,22**). IL-6 released by the endothelial cells could enhance the previously mentioned effects, and IL-8, as it was described by Abbas *et al.*(**1**), is an activating and chemotactic factor for polymorphonuclear leukocytes, mainly for neutrophils.

Neutrophils have the properties of chemotaxis, adherence to immune complexes and phagocytosis. This latter possesses oxidative and non-oxidative(**13**) mechanisms. In addition, polymorphonuclear leukocytes can be activated by TNF-α released by the mononuclear phagocytes Thus, venom could also act as an indirect inducer of neutrophils via TNF-α .

Therefore, we can hypothesize that venom can trigger various host defense mechanisms. Rejection of tumor cells observed with *Bothrops jararaca* venom may be the result of one or more mechanisms above described. Further studies will contribute to a better understanding of these phenomena, as well as confirm or not the proposed hypothesis.

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✉ **CORRESPONDENCE TO:**
R. J. DA SILVA - Caixa Postal 577, CEP 18.618-000, Botucatu, São Paulo, Brasil.

Caixa Postal 577
18618-000 Botucatu SP Brazil
Tel. / Fax: +55 14 3814-5555 | 3814-5446 | 3811-7241
e-Mail
jvat@cevap.org.br